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14. ABSTRACT

The long-term objective of this research is to develop a device for treating spinal cord injury. The specific aims of the proposed study are to test new types of collagen biomaterials. Moreover we will be investigating the effects of incorporating genes from nerve growth factors into the collagen scaffolds and seeding the scaffolds with neurogenic cells. The standardized defect site is a 5-mm gap in the rat thoracic spinal cord. Our principal method of evaluation is histomorphometry. During the past project year the following were accomplished, toward achieving the objectives of determining the effects of selected design variables on the reparative processes in spinal cord defects: 1) investigation of nanoparticles, as carriers of genes for neurotrophic factors, to be incorporated into collagen scaffolds for implantation into spinal cord defects; 2) development of an injectable collagen formulation as a carrier for plasmid DNA encoding for neurotrophic factors; 3) formulation of collagen scaffolds containing hyaluronic acid; and 4) investigation of the conditions *in vitro* for the neuro-differentiation of neural stem cells.

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Spinal cord, rat, collagen, tube, regeneration, axons

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I. INTRODUCTION

The long-term objective of this research is to develop a device for treating spinal cord injury. The specific aims of the proposed study are to test new types of collagen tubes and porous collagen scaffolds. Moreover we will be investigating the effects of incorporating genes from nerve growth factors into the collagen scaffolds and seeding the scaffolds with marrow-derived mesenchymal stem cells. The standardized defect site is a 5-mm gap in the rat thoracic spinal cord. Our principal method of evaluation is histomorphometry.

Our supposition is that an appropriate synthetic substrate (*i.e*., the collagen scaffold) will mimic or perhaps improve upon the documented ability of peripheral nerve grafts to promote the regrowth of injured spinal axons, and that the seeded stem cells will differentiate under the influence of the endogenous regulators to a support cell phenotype. Collagen-based materials are the bio-inspired biomaterials being used for the fabrication of the tube, covering film and porous scaffold.

II. BODY

During the past project year, research focused on the following:

1. Novel magnetic calcium phosphate nanoparticles were developed as non-viral vectors for transfection of cells with the genes for neurotrophic factor.
2. Stem cells were transfected with the genes for growth factors which hold may enhance recovery to spinal cord injury, incorporated into a collagen scaffold: an anti-angiogenic factor, endostatin; and bone morphogenetic protein-7.
3. Collagen-hyaluronic acid composite scaffolds were developed for neural tissue engineering.

The following describes the achievement related to the development of navel calcium phosphate nanoparticles and hyaluronic acid-collagen composite scaffolds.

A. Novel Magnetic Calcium Phosphate Nanoparticles as Non-Viral Vectors

1. Background

The goal was to employ nanoparticles to deliver genes for neurotrophic factors to spinal cord injury. Challenges in employing NPs for the delivery of therapeutic agents include their targeting to, and their retention at, a specific site. One recent development which has the promise of meeting these challenges, so that the potential of NP drug and gene delivery can be realized, is the magnetic control of the particles [1-3]. Superparamagnetic NPs (mNPs), whose location can be precisely directed and maintained by an external magnet, have been of recent interest for numerous applications including non-viral vectors. Studies have demonstrated that an external magnet can attract magnetic particles, with the potential of targeting anticancer drugs to a tumor [4,5]. The term magnetofection was coined to describe the action of a magnetic force on gene vectors combined with magnetic particles [3]. These magnetic vectors have been exclusively based on iron oxide for which there has been some concern related to cytotoxicity [6].

Prompted by the need to develop safe and effective magnetic NPs, we developed a well-controlled, wet-chemical method for producing magnetic calcium phosphate, *viz*., hydroxyapatite (Hap), NPs as non-viral vectors. Hap, $Ca_{10}(PO_4)_6(OH)_2$, is the principal form of calcium phosphate in the body—the mineral constituent of hard tissues, being stable at neutral pH. Hap is commended as a biomaterial based on its biocompatibility and biodegradability [7], and has been well-studied and used as delivery vehicle for proteins [8] and genes [9,10].

The objective of the present study was to to produce magnetic synthetic Hap (mHap) NPs and magnetic natural bone mineral (mNBM) NPs as non-viral vectors for the plasmid DNA

(pDNA) encoding a potent neurotrophic growth factor, glial cell line-derived neurotrophic factor (GDNF). GDNF is one of several neurotrophic factors which regulate many critical aspects of the ontogeny of neurons, such as the number of neurons in a given population, neurite branching and synaptogenesis, adult synaptic plasticity and maturation of electrophysiological properties [11]. Specific aims were to investigate the structure and properties of the mNPs, and the temporal expression of GDNF by rat marrow stromal cells (also referred to as mesenchymal stem cells, MSCs) transfected *in vitro* by the mNPs under the action of a magnet.

2. Materials and Methods

Hap crystallites were prepared by the precipitation method previously described [12]. In brief, orthophosphoric acid solution was added into dispersed calcium hydroxide suspension at a rate of 1.5 mL/min. and aged for 20 hrs. The entire process was carried out at 80°C in a hot water bath. The final calcium:phosphorous ratio was 1.67 to match the stoichiometric ratio in Hap. The pH value was adjusted to 8 by the addition of ammonium hydroxide (NH_4OH) during the precipitation process. The term, "nanoparticles," was adopted to describe the crystallite aggregates, which were from tens to hundreds of nanometers in diameter.

Iron (II) chloride solution was added to the dispersed suspensions of the Hap and NBM (anorganic bovine bone, Geistlich Biomaterials, Wolhusen, Switzerland) NPs, at rate of 1.5 mL/min. The pH value was again adjusted to 8 by adding NH_4OH at 80°C, and the suspension aged for another 10 hours at 80°C. Subsequently, the precipitates were washed with deionized water three times. Finally, the aqueous suspensions were freeze-dried to form a dispersible powder. As a control, magnetite was precipitated from the iron chloride solution by adjusting the pH to 8 using NH_4OH at 80°C, and the suspension aged for another 10 hours at 80°C.

The calcium, phosphorous, and iron content of the NPs was determined by an inductively coupled plasma optical emission spectrometry. The crystalline structure of the NPs was analyzed using x-ray diffraction (XRD) and the morphology of the NPs was observed by scanning electron microscopy (SEM). For ultrastructural studies, NP samples were deposited on carbon-coated copper grids from aqueous solution and air-dried. The particle size of the NPs was determined from dynamic light scattering (DLS) measurements. The magnetic properties of the mNPs were analyzed by a superconducting quantum interference device (SQUID).

The plasmid encoding rat GDNF (pGDNF) was obtained from Children's Memorial Hospital, Chicago, Illinois. The pDNA was mixed into a dispersion of the non-magnetic and magnetic Hap and NBM NPs (mNPs), and magnetite NPs, at 3 different weight ratios, and incubated for 15 minutes in order to allow DNA to bind to NPs to form pDNA-NP transfection complexes. The mNPs were removed from the suspension by attraction to a neodymium iron boron magnet. The suspensions of non-magnetic Hap and NBM complexes incorporating the pDNA were separated by centrifugation.

Rat MSCs were harvested from both the femurs and tibiae of 6 young Lewis rats ($<$ 6 weeks old) as previously described [13]. The cells from the 6 rats were cultured separately throughout the experiment. For transfection, equal volumes of pDNA (25 μg/mL) and magnetic NPs (1 mg iron/mL) were mixed together and allowed to set for 15 min. to form transfection complexes. 3.2×10^4 MSCs of passage 2-4 were cultured in 24-well (2 cm^2) culture plates and grown in 1 mL of expansion medium. After 24 hr. of culture, the medium in the wells was replaced with 250 μl serum-free medium containing the transfection complexes (0.625 μg pDNA/well), for the 4-hr. transfection period. Magnetofection was performed by applying a magnetic field during the first 15-min. stage of the 4-hr. transfection period. A neodymium iron

boron magnet was placed under the culture dish. After the 4-hr. transfection period, 250 μL of expansion medium was added to each well, and the dishes incubated for an additional 24 hr. The supernatant was then replaced with fresh expansion medium.

Twenty-four hours post-transfection, the culture medium was refreshed, and the medium collected every 3 days thereafter at medium changes on (at days 4, 7, 10, and 13; n=5) for enzyme-linked immunosorbent assay.

3. Results

Nanoparticles (NPs) of synthetic hydroxyapatite (Hap) and natural bone mineral (NBM) were rendered magnetic by treatment with iron ions using a wet-chemical process. The magnetic NPs (mNPs), which were about 300 nm in diameter, displayed superparamagnetic properties in a superconducting quantum interference device, with a saturation magnetization of about 30 emu/g. X-ray diffraction and transmission electron microscopy revealed that the magnetic properties of the NPs were the result of the hetero-epitaxial growth of magnetite on the Hap and NBM crystallites.

The Hap NPs adsorbed virtually all of the pDNA from its solutions at the highest NP:pDNA (by wt.) ratio. In comparison about 90 % of the pDNA was bound by the NBM NPs at the highest NP:pDNA (by wt.) ratio. As might have been expected, the percentage of pDNA bound by the samples increased with the ratio of NP:pDNA. The percentage of pDNA bound by the Hap and NBM NPs remained about the same after addition of the iron ions to form the magnetic NPs. In contrast, the magnetite samples displayed poor affinity for binding pDNA, compared to the Hap and NBM NPs despite the fact that the small size of the magnetite crystallites would have indicated that the number of magnetite particles and their surface area would have been far greater than the mHap and mNBM.

Virtually all of the cells undergoing magnetofection with mHap and mNBM expressed GFP (Fig. 1a and b). In comparison, it was estimated that 60-70% of the cells transfected without magnetofection displayed the green fluorescence reflective of GFP expression. Also of note was the presence of particulate deposits in the cytoplasm of the cells (Fig. 1a-d), consistent in appearance with the NPs. These particles were not seen in the cultures without the NPs.

No GDNF was detected in the medium from the non-transfected MSC control cultures. All of the mNP groups demonstrated overexpression and secretion of GDNF at each of the collection periods (Fig. 2). For all of the groups the rate of production of GDNF decreased after 7 days (Fig. 2). For the cultures that were not exposed to magnetofection, the mNBM NPs resulted in substantially higher GDNF expression at the 3-day collection periods ending on days 4 and 7 (Fig. 2): 2-fold higher at 4 days and 37% higher at 7 days. Of note was that the 15-min. application of the magnet significantly increased the expression of GDNF for both the mHap and mNBM. For mHap, magnetofection increased GDNF levels: 2.3 times at 4 days; 82% increase at 7 days; and 65% increase at 10 days. For mNBM, the magnetofection had the effect of increasing GDNF by: 23% at 4 days; 33% at 7 days; and 22% at 10 days. There were no notable differences in the GDNF expression under the influence of magnetofection for the mHap versus the mNBM NPs at any of the collection periods. The levels of expression in the mNP groups undergoing magnetofection were about 50% of the values measured in the cultures treated with the lipid transfection reagent (Fig. 2). There was no noticeable difference in the GDNF expression in the GP groups with the two pGDNF levels (0.625 versus 2 μg/well; Fig. 6).

Three-factor ANOVA revealed the significance of the effects of NP type ($p<0.0001$; power=1), magnetofection ($p<0.0001$; power=1), and collection period ($p<0.0001$; power=1) on

the concentration of GDNF in the medium. Fisher's PLSD post-hoc testing demonstrated that the differences in GDNF levels between mHap and mNBM, and between groups with and without magnetofection, were highly significant. All of the collection period comparisons were highly significant except for the comparison of the data from days 4 and 7, which was not significant. One-factor ANOVA of the GDNF data at the 4-day and 7-day periods separately, revealed that the GDNF levels for the mNBM NPs with ($p=0.0002$; power=1) and without ($p<0.0001$; power=1) magnetofection were statistically significantly higher than the respective values for the mHap NPs. For the 3-day collection ending on day 7, only the difference between the mNBM and mHap NPs without magnetofection was statistically significant ($p<0.0001$; power=1). Two-factor ANOVA of the GP data demonstrated that there was a significant effect of collection period ($p<0.0001$; power=1), but no effect of pGDNF concentration ($p=0.57$; power=0.09) on GDNF expression.

The accumulated amount of GDNF for the cultures treated with pGDNF-mNBM NPs without application of the magnetic field, calculated by summing the amounts of GDNF from each of the collection periods, reached about 1.9 ± 0.04 ng/ml (mean ± standard deviation). This value was 40% higher than that from the cultures treated with the mHap NPs. Application of the magnetic field increased the accumulated GDNF concentration in the mHap cultures from 1.4 ± 0.02 ng/ml to 2.4 ± 0.07 ng/ml, for an increase of 70% and in the mNBM cultures from 1.9 ± 0.04 ng/ml to 2.4 ± 0.06 (26% increase). Two-factor ANOVA revealed significant effects of NP type ($p<0.0001$; power=1) and application of the magnet ($p<0.0001$; power=1) on the accumulated GDNF. Separate one-factor ANOVA for the data without application of the magnet showed that the difference in GDNF for the mHap and mNBM groups was highly significant ($p<0.0001$; power=1). There was no statistically significant difference in the GDNF levels for the mHap and mNBM cultures to which the magnet was applied ($p=0.7$).

3. Discussion

The notable finding of this work was that NPs comprising synthetic Hap and NBM crystallites could be modified to be made magnetic and that these mNPs displayed enhanced gene transfection when used as non-viral vectors under the action of a magnetic field. Magnetofection resulted in an approximate 2-fold increase in gene expression through 7 days post-transfection for the mHap NPs. The percentage increase in GDNF expression resulting from the 15-min. period under the influence of the magnetic filed was not as high for the mNBM NPs, but significant at the first 3 collection periods (from 20-30% higher than non-magnet controls). Also of note was that the LDH assay showed that there was no cytotoxicity of the mNPs.

While there are questions concerning the intrinsic biocompatibility of magnetic metallic NPs, calcium phosphate NPs are widely commended as non-viral vectors based on their: DNA binding affinity; protection of pDNA from enzymatic degradation; favorable safety profile; and biodegradability [14,15]. Of interest in the current experiments was the difference in the GDNF tranfection resulting from the synthetic, mHap, and natural, mNBM, NP vectors, in cultures without application of the magnet during the transfection period. The mNBM yielded a 40% higher GDNF level. This difference in transfection, between mNBM and mHap NPs, was overcome by the application of the magnetic field.

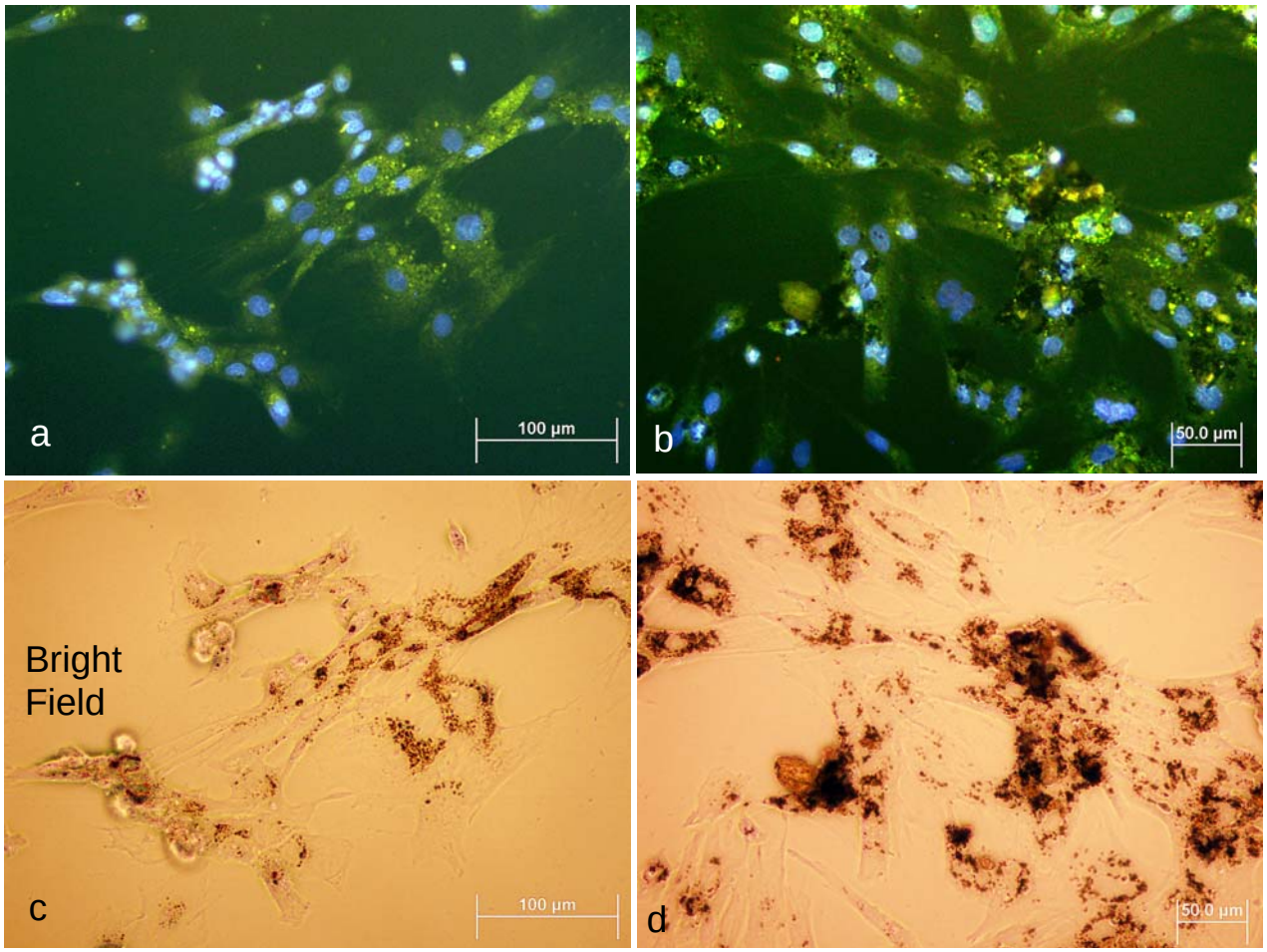

Fig. 1. Micrographs of the same fields of view for MSC cultures transfected by pGFP-GDNF mHap (a, c) and pGFP-GDNF mNBM (b, d), 6 days post magnetofection. (a-b) Fluorescence micrographs of cultures showing GFP, with the nuclei stained with Hoechst 33342 nuclear stain. (c-d) Bright field micrographs

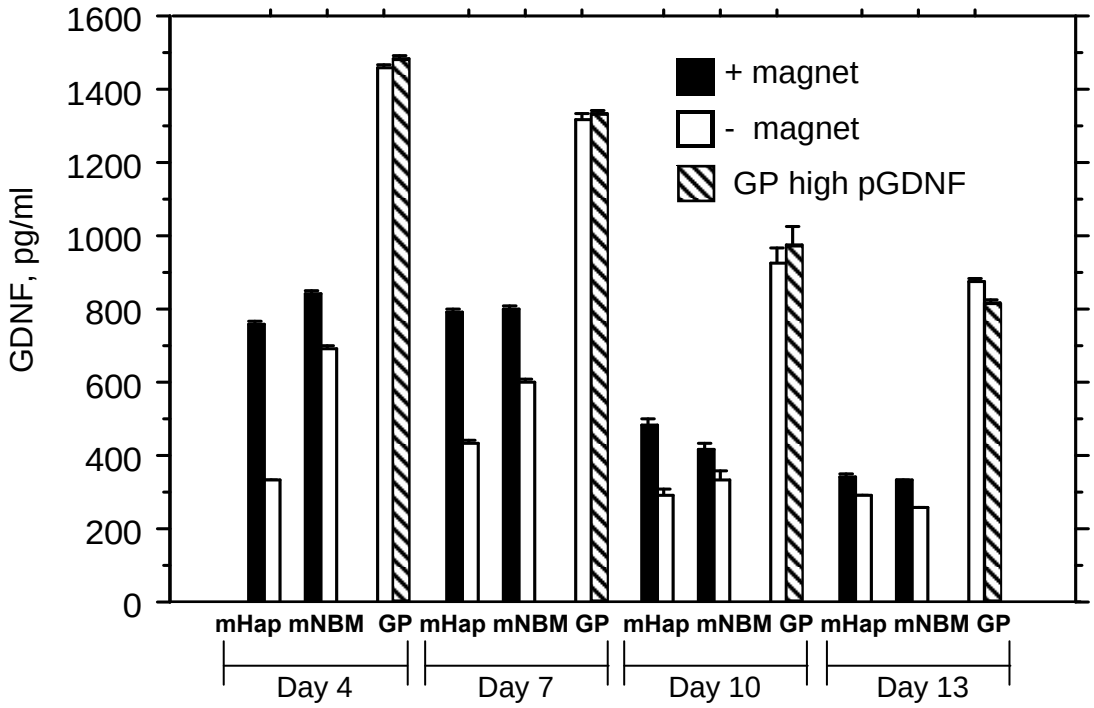

Fig. 2. The GDNF in the medium of cultures of MSCs transfected by mHap and mNBM, with and without application of the magnet. Also shown are the GenePORTER (GP) controls run at the same dose of pGDNF per well (0.625 µg pDNA/well; white bar) and a higher dose (2 µg pDNA/well). Mean±SEM; *n*=5.

The initial goal of the current study was to attempt to replace iron oxide for magnetofection with calcium phosphate NPs rendered magnetic by the addition of iron ions. Instead of achieving this goal, we produced calcium phosphate NPs with adherent magnetite crystallites. While falling short of our objective, the mHap and mNBM NPs do provide improved pDNA binding and the benefits of the properties of the Hap and NBM, and importantly reduce the amount of iron oxide that would be introduced into the body.
The 2-week accumulated levels of GDNF of about 2 ng/ml in the magnetofection cultures with the mHap and mNBM NPs containing as little as 0.6 μg plasmid/well in 24-well plates compared with values of approximately twice that value for the same amount of plasmid incorporated into a commercially-available lipid transfection reagent. Of importance is that the level of GDNF expression by rat MSCs following magnetofection was high enough to result in GDNF concentrations which were found to be close to therapeutic concentrations in previous *in vitro* investigations: 1 ng/ml increased by nearly 100% the number of trigeminal ganglion sensory neurons in culture at 5 days post-plating [16]; and 10 ng/ml nearly doubled dopamine neuron survival and reduced the rate of apoptosis from 6% to 3% in human embryonic dopamine neurons cultures [17]. While lipid transfection reagents may result in higher expression levels there remain concerns regarding their safety profile [18,19].

B. Hyaluronic-Collagen Composite Scaffolds

A hyaluronic acid–collagen (HA-Coll) sponge with an open porous structure and mechanical behavior comparable to brain tissue was developed [20].

1. Background

Coll and HA are widely used natural polymers for the development of implantable matrices. Type I collagen, a major structural protein of extracellular matrix, supports the growth of a wide variety of tissues, while its structure imparts favorable properties such as mechanical strength [21]. HA (also referred to as hyaluronan) a high molecular weight GAG polymer composed of repeating units of D-glucuronic acid and n-acetyl-D-glucosamine, is a ubiquitous biopolymer in the mammalian body and one of the principal components of brain extracellular matrix [22,23]. The favorable attributes of HA have led to the preparation of HA hydrogel biomaterials [24-26], and the combination of HA and Coll as a tissue engineering scaffold [27-29].

2. Materials and Methods

HA-Coll scaffolds with different mixing ratios were prepared by a freeze drying technique, and crosslinked with water soluble carbodiimide to improve mechanical stability. The pore structure of the samples was evaluated by light and scanning electron microscopy, and the mechanical behavior was analyzed by mechanical compression and tension testing. The degree of crosslinking was determined by the water absorption and trinitrobenzene sulfonic assay, and the HA content was determined by a carbazole assay.

Neural stem cells (NSCs) were isolated from the brains of 7 rats and cultured in 2D and in 3D cultures. In order to investigate the effects of the scaffold type on the support of the NSCs and their subsequent differentiation, NSCs were inoculated on the different types of scaffolds: pure Coll, HA-Coll (Coll:HA=1:2), and pure HA sponges. The Coll:HA=1:2 scaffold was selected for cell seeding on the basis of its mechanical behavior which was substantially different from the collagen scaffold. In brief, the neurospheres that formed in the T-75 dishes and the

dissociated cells (5×10^5 cells/cm^2) were collected and seeded into the scaffolds in induction medium for neuronal maturation. For cell seeding onto the 3D scaffolds, the dry matrix specimens were rinsed with PBS and then incubated in culture medium for 2 hrs. for pre-wetting followed by air-drying to favor cell attachment. A 100 µl suspension containing 2.5×10^5 passage 2 to 3 cells and neurospheres was pipetted onto each 8-mm diameter scaffold (n=7) in agarose-coated wells. After 30 mins., medium was added to the cultures to total 0.5 ml. Medium was changed every other day. The cultures were terminated after 2 weeks. Immunohistochemistry was employed to evaluate the differentiation of the NSCs to neuronal cells.

3. Rssults

The results showed that HA-Coll scaffolds containing an open porous structure with homogeneous pore size distribution could be fabricated. The percentage porosity of the samples, which ranged from 75-91%, increased (Table 1), and the apparent densities decreased (Table 1), with increasing HA content. The percentage porosity of the pure Coll and HA scaffolds, and the brain tissue were comparable (Table 1). The degree of crosslinking gradually decreased with the increasing amount of HA, from nearly 90% down to around 50% (Table 1). The addition of HA to the collagen scaffolds increased the water uptake capability, because of the hygroscopic nature of HA (Table 1). All the sponges absorbed the water within 30 sec. and were saturated within 5 min. The pure HA scaffolds displayed a 60-fold increase in weight as a result of water uptake, which was about 50% higher than the HA-Coll samples (Table 1). There were strong inverse linear relationships between the water absorption with the degree of crosslinking for the type I and type II HA-Coll scaffolds: R^2=0.95 and R^2=0.89, respectively by linear regression analysis. This was reasonable because in the crosslinking process the hydrophilic functional groups were consumed to react with carbodiimide crosslinking agent.

The compressive stress-strain curves for the hydrated scaffolds displayed the typical characteristics of low-density, open-cell foams [21]. Distinct linear elastic, collapse plateaus and densification regimes were observed in all cases. The pure type I and II Coll scaffolds displayed similar compressive behavior, with the type II scaffolds displaying about 15% lower strength and modulus (Table 1). Addition of HA to the type I and II Coll scaffolds substantially reduced compressive strength and modulus (Table 1). The values for the HA-Coll scaffolds with Coll:HA=1:2 were less than 50% of the values for the respective collagen scaffolds. (Table 1). The stress-strain behavior of the brain tissue was most like that of the pure HA scaffold. The compressive modulus of brain tissue (1.24 kPa) fell between the values for the pure crosslinked HA sponge and the Coll:HA=1:2 group (Table 1).

Of note was a strong linear correlation between the compressive modulus with the apparent density for the type I HA-Coll scaffolds, R^2=0.99, and a modest correlation for the type II HA-Coll scaffolds, R^2=0.76, by linear regression analysis.

The tensile stress-strain curves for the collagen and HA-Coll scaffolds increased to a peak value and then dropped to zero. The behavior for the brain tissue in tension differed from the scaffolds in that the stress decreased gradually to zero after the peak value was reached. Moreover, whereas the compression behavior of the type I and II scaffolds was similar, the peak tensile strength of the type I scaffold was about 2-fold higher than for the type II scaffold (Table 1). While addition of HA resulted in a substantial decrease in the peak tensile strength of the type I collagen scaffolds (by >2.5-fold), the addition of HA to the type II samples slightly increased the peak tensile strength (Table 1). The tensile modulus of elasticity of the type II

scaffold was about 10% less than the modulus of the type I scaffold (Table 1). Addition of HA to both the type I and type II scaffolds greatly decreased (by 6- to 7-fold) the tensile modulus of the samples (Table 1).

The cell-seeded HA scaffolds displayed signs of fragmentation at the end of the 2-week culture period. While cells staining for neuronal cell markers could be seen distributed through the Coll, HA-Coll, and HA scaffolds (Fig. 3), the cell distribution was different among the groups. In HA-Coll and HA scaffolds (Fig. 3D and 6G), cells appeared to assemble into clusters displaying the typical features of neurospheres. In contrast, cells in the collagen scaffolds were dispersed sparsely and did not form neurospheres (Fig. 3A). Nestin, a neurospecific intermediate filament protein [30], was prominent in cells in the three different groups of scaffolds. The intensity of expression and density of MAP2, which is used for labeling of dentritic trees of mature neurons, was much higher in the HA-Coll samples than in the HA scaffolds (Fig. 3E and 3H). In comparison there was little expression of MAP2 by cells in the Coll scaffolds (Fig. 3B). The neuron-specific nuclear protein, NeuN, was also expressed prominently surrounding the nuclei in the cells in HA-Coll and HA scaffolds (Fig. 3E and 6H). Tuj-1, beta III tubulin, was found exclusively in the neuronal processes in all the groups (Fig. 3C, 3F, 3I). The co-localization of nestin, Tuj-1, as well as DAPI proteins with nucleus was revealed in Fig. 3F and 3I.

4. Discussion

The results of this study provide a basis for the preparation of HA-Coll scaffolds for neural tissue engineering. While HA is the major extracellular matrix component of adult central nervous system tissues, and collagen a lesser component if even present, there are several potential benefits of an HA-Coll scaffold for neural tissue engineering. The principal benefits of incorporation of collagen in HA scaffolds relate to: 1) the control that it offers over strength, stiffness, and degradation rate; and 2) the fact that collagen may provide ligands for the integrins of neuronal support cells necessary during the regeneration process. Of potential relevance is that prior studies have found that embryonic spinal cord epithelium [31] and chick neural retina tissue [32] synthesize type II collagen, indicating that collagen in addition to HA may play an important role in the formation of select nervous tissues. These findings also underlie the rationale for the ultimate comparison of the performance of type I and II collagens for neural applications.

The findings of the current work demonstrate the effects that the increasing content of HA have on the physical and mechanical properties of the HA-Coll composite scaffolds prepared using the same freeze drying protocol. This work serves as a guideline for how the processing (*viz*., freezing) conditions may have to be changed to produce select HA-Coll scaffolds with properties to meet certain design specifications. Including brain tissue as a comparative control in this investigation provided the opportunity to begin to determine how best to prepare an HA-Coll scaffold to match selected physical and mechanical properties of the brain. While the pure HA scaffold came closest to replicating the mechanical behavior of brain tissue, those scaffolds may not ultimately prove to have sufficient handling properties for a tissue engineering approach to treating defects in the brain.

The most notable difference between the type I and II Coll scaffolds in this study related to the mechanical behavior. The type I Coll scaffolds were 20% higher in compressive yield strength and 93% higher in peak tensile strength. The mean value of the compressive modulus of the type I Coll samples (6.3 kPa) fell within the range of values previously reported for

collagen-GAG scaffolds of comparable pore characteristics crosslinked with EDAC [21]. Addition of HA to the Coll scaffolds provided the methodology to vary the compressive and tensile moduli (and stiffness) over wide ranges.

A notable finding of the present study was high linear correlation between compressive modulus and apparent density for the Coll and HA-Coll scaffolds, as was found in prior studies of porous collagen scaffolds [21], deviating from the squared dependence predicted by open cell foam theory [33]. In our study, the reason that the dependence of modulus on density did not follow foam theory is likely that the mechanical properties of the struts comprising the scaffolds of varying density changed with the Coll:HA ratio.

5. Summary

Complexing HA with type I and II Coll enables the fabrication of scaffolds with a range of physical and mechanical properties suitable for neural tissue engineering applications, involving the treatment of defects in the brain. The compressive modulus increases linearly with the HA content of the specimens. HA and HA-Coll scaffolds, which parallel certain mechanical n=behavior of brain tissue, favor the differentiation of NSCs to neuronal cells *in vitro*.

Table 1. Properties of the Scaffolds (Mean±SEM)

Sample[a]	% Porosity (n=6)	Apparent Density, g/cm^3 Mean (n=6)	Degree Crosslinking % (n=4)	Water Absorp., times inc. (wt). (n=6)	Compression (n=6)		Tension (n=6)	
					Yield Strength (5% strain), Pa	Modulus (5% strain), kPa	Peak Strength, Pa	Modulus (5% strain), kPa x10-2
(A) Type I Coll	75±8	0.0335	88±0.4%	26.6±0.4	315±16	6.31±0.33	2.7±0.1	90±3
(B) I:HA=2:1	84±2	0.0311	81±1.1%	31.4±0.8	246±8	4.91±0.17	NA	NA
(C) I:HA=1:1	88±1	0.0276	71±2%	34.3±0.3	176±17	3.52±0.35	NA	NA
(D) I:HA=1:2	91±1	0.027	57±0.6%	36.7±0.5	136±29	2.72±0.57	1.0±0.1	13±1
(E) Type II Coll	76±1	0.0322	81±0.4%	26.4±0.4	262±41	5.24±0.82	1.4±0.1	81±1
(F) II:HA=2:1	84±0.4	0.0262	71±0.9%	33.1±0.7	136±24	2.72±0.48	NA	NA
(G) II:HA=1:1	90±2	0.023	62±3%	36.3±0.5	116±17	2.32±0.33	NA	NA
(H) II:HA=1:2	91±1	0.0174	49±0.8%	38.2±0.7	93±25	1.86±0.50	1.8±0.1	12±1
(I) HA	80±1	0.0202	17±4	62.6±1	67±10	1.33±0.20	0.23±0.02	8±1
(J) Brain tissue	76±1	0.0252[b]	NA	NA	53±8	1.06±0.17	0.30±0	13±0.4

[a] Letters refer to samples in Figs. 2 and 5.

[b] Value of hydrated cortical samples divided by volume.

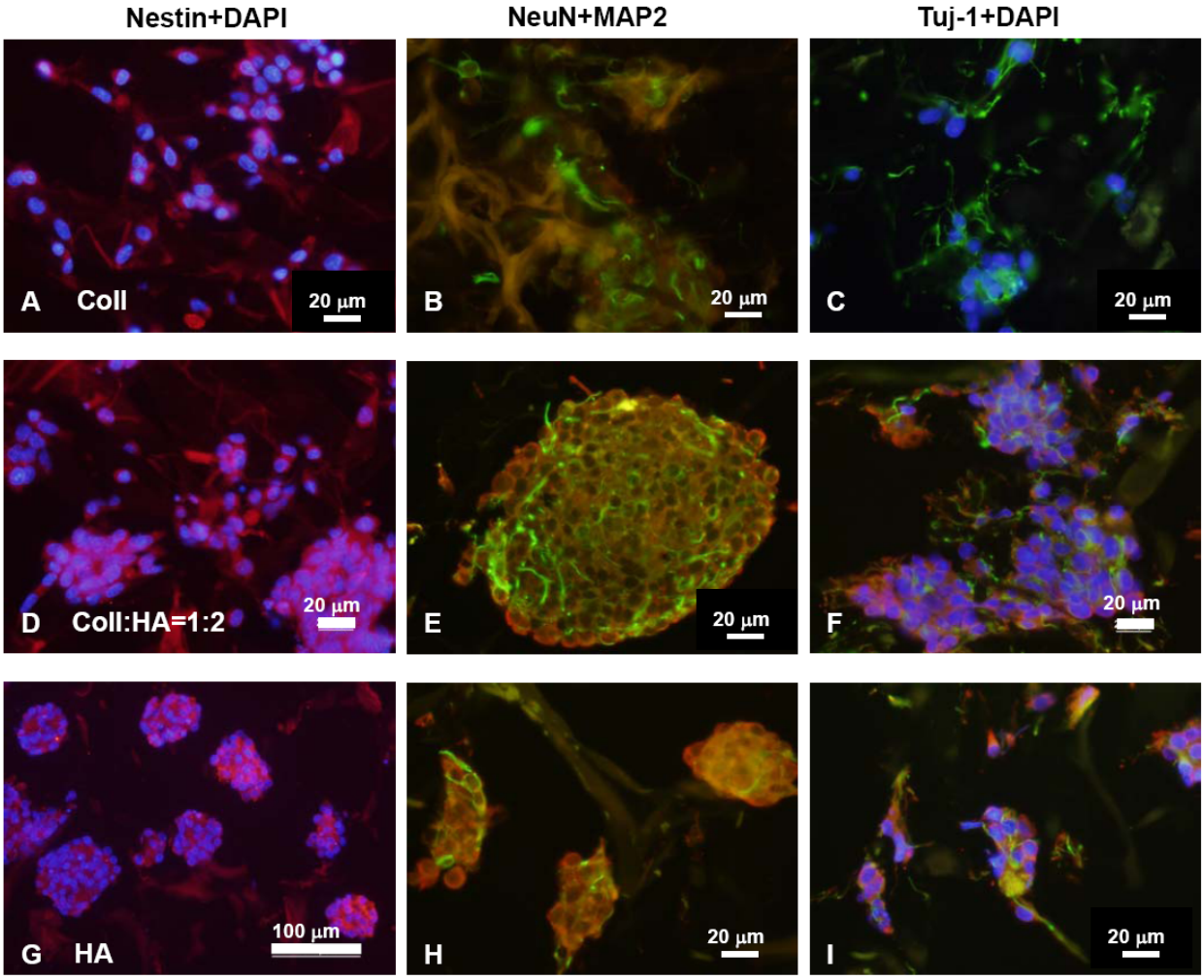

Fig. 3. Immunochemistry of neural stem cells grown in scaffolds in neurogenic medium, 2 weeks after seeding. The nucleus was stained blue with DAPI in blue. Neurospecific intermediate filament protein (Nestin) and neuron-specific nuclear protein (NeuN) are in red, and tubulin β III (Tuj-1) and major microtubule associated protein (MAP 2) are in green. (A-C) type I Coll scaffold; (D-F) HA-type I Coll scaffold with Coll:HA=1:2; and (G-I) HA scaffold.

III. KEY RESEARCH ACCOMPLISHMENTS

- Novel magnetic calcium phosphate nanoparticles were developed as non-viral vectors for transfection of cells with the genes for neurotrophic factor
- Stem cells were transfected with the gene for an anti-angiogenic factor, incorporated into a collagen scaffold
- Collagen-hyaluronic acid composite scaffolds were developed for neural tissue engineering

IV. REPORTABLE OUTCOMES

Bolliet C, Bohn MC, and Spector M. Non-viral delivery of the gene for glial cell line-derived neurotrophic factor to mesenchymal stem cells *in vitro* via a collagen scaffold. Tissue Engr. 2008;14:207-214.

Sun X-D, Jeng L, Bolliet C, Olsen BR, and Spector M. Nonviral endostatin plasmid transfection of mesenchymal stem cells via collagen scaffolds. Biomaterials 2009;30:1222–1231.

Wang T-W, Spector M. Development of hyaluronic acid-based scaffolds for brain tissue engineering. Acta Biomaterialia (In press)

V. CONCLUSIONS

These results demonstrated the potential utility of nanoparticles, injectable collagen, and collagen scaffolds for the formulation of implants to treat defects in the spinal cord.

VI. REFERENCES

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VII. APPENDICES

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